Exhibit A
Reyn and Bentzon, *WHO Bull.*, 14:567-576, 1956

[Please see attached six pages]

RÉSUMÉ

Les auteurs ont déterminé la concentration en toxine alpha des Préparations internationales de Référence des Sérums anti-*Clostridium welchii* B et D, qui servent de référence pour les sérums antitoxiques béta et epsilon respectivement. Les sérums anti-*Cl. welchii*, types B et D, ont été soumis aux essais par rapport à deux toxines d'épreuve de *Cl. welchii* type A, dont les teneurs en toxine alpha avaient été établies par comparaison avec l'Etalon international de Sérum antitoxique alpha.

La toxine alpha est une lécithinase qui est létale, hémolytique, et capable de produire un trouble lorsqu'elle est mise en incubation avec la lécithovitelline dans une solution de jaune d'œuf. Trois méthodes ont été employées pour déterminer l'excès de toxine par rapport à l'antitoxine : mort de la souris (test L+), hémolyse des globules rouges (test Lh), trouble de la solution de jaune d'œuf (test Lv).

La moyenne des résultats de toutes les épreuves indique que la Préparation internationale de Référence de Sérum anti-*Cl. welchii* type B contient 282 unités antitoxiques par ml et que la Préparation internationale de Référence de Sérum anti-*Cl. welchii* type D contient 93 unités antitoxiques par ml.

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USE OF SYNTHETIC, CRYSTALLINE, L-α-DIMYRISTOYL LECITHIN IN CARDIOLIPIN ANTIGENS

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SYNOPSIS

Experiments were carried out by the authors to determine whether synthetic, crystalline, L-α-dimyristoyl lecithin could replace natural purified lecithins in the preparation of cardiolipin antigens. These experiments were designed specifically to find out whether it was possible to obtain the same serological reactions, qualitatively and quantitatively, with the test antigen as with a reference antigen containing natural lecithin, and whether the test antigen had the same keeping qualities as the reference antigen.

The tests used were the quantitative complement-fixation test as modified by Mørch in 1933, and the VDRL slide flocculation test.

The results showed that synthetic, crystalline, L-α-dimyristoyl lecithin could replace natural lecithin in the preparation of cardiolipin antigens, but that the antigens prepared with the synthetic lecithin were significantly less sensitive than those prepared with an equimolar amount of natural lecithin. The authors consider that further investigation is required before the use of synthetic lecithin is finally adopted.

Cardiolipin antigens, used in serological tests for syphilis, are mixtures of the sodium salt of the phospholipid, cardiolipin, with purified natural lecithin (beef-heart or egg) and usually also with cholesterol.

Even highly purified natural lecithins consist of a mixture of individual lecithins containing chiefly unsaturated, but also some saturated, fatty acids.

The most recent modification in the preparation of cardiolipin antigens is the introduction of synthetic lecithin.[2, 3, 4] Several types of synthetic lecithin have been produced [18] and have been found to be serologically active to a varying degree.[6, 8, 15, 18] However, the first batches of synthetic lecithin were not quite satisfactory with respect to either the keeping

qualities or the sensitivity of the antigens and antigen suspensions.[6, 18] Evidence is now accumulating that one of the latest products—a crystalline, L-α-dimyristoyl lecithin [1]—may replace the highly purified natural lecithins [13, 14] in the preparation of cardiolipin antigen.[9, 10, 11, a] Also, an unsaturated, natural lecithin has recently been reported to be useful.[19]

The complete knowledge of the constitution of synthetic products opens up the possibility of a closer analysis of the mode of combination in, for example, syphilitic sera between the antigen lecithin and the antibodies (reagins).

In serological experiments the significance of the type and degree of hydrogenation of the fatty acids may also be submitted to a closer analysis.[5]

For practical as well as for experimental purposes important requirements of the synthetic products are constancy, purity, and relatively low cost.

Before introducing a new substance into the preparation of cardiolipin antigens, it is important to investigate:

(1) whether it is possible to obtain the "*same serological reactions*", qualitatively and quantitatively, with a test antigen containing, for example, synthetic lecithin, as with a reference antigen containing natural lecithin;

(2) whether the test antigen has the *same keeping qualities* as this reference antigen.

In the present paper, quantitative serological experiments carried out to evaluate the above points in the case of synthetic, crystalline, L-α-dimyristoyl lecithin are described.

Materials

In December 1953, 400 mg of synthetic, crystalline, L-α-dimyristoyl lecithin were received from Professor E. Baer, Toronto, Canada, following a request by the World Health Organization for a serological testing at the WHO International Serological Reference Laboratory, Statens Serum-institut, Copenhagen. The substance was kept in a dry condition at about + 4° C until 8 February 1954, when a 3% solution in absolute ethanol was prepared. The lecithin was readily soluble in ethanol and the resulting solution was clear and colourless.

Antigens

On 9 February 1954, four antigens were prepared—two complement-fixation antigens (CF) and two VDRL slide flocculation antigens; the International Reference Preparations for 1953 (IRP 1953) were used as references. The antigens were stored at room temperature. The composition of the various antigens is given in Table I.

[a] See article by M. Faure & C. de la Vaissière on page 577 of this number of the *Bulletin*.

TABLE I. COMPOSITION OF ANTIGENS

Antigens	Lecithins (w/v %)*	Cardiolipin IRP 1953 (w/v %)*	Cholesterol Pfanstiehl precipitated from ethanol (w/v %)*
	Synthetic lecithin		
CF: test	0.078	0.0175	0.3
VDRL: test	0.255	0.03	0.9
	Egg lecithin IRP 1953		
CF: reference	0.0875	0.0175	0.3
VDRL: reference	0.27	0.03	0.9

* weight/volume: w/v %
weight: lecithin, cardiolipin, or cholesterol
volume: absolute ethanol

The molecular weight of the synthetic lecithin—namely, synthetic dimyristoyl-L-α-glyceryl-phosphoryl-choline—was calculated [3] at 695.6 and the molecular weight of egg lecithin was calculated at 785 (P = 3.95%).[14] The test and reference antigens were thus comparable with respect to content of lecithin, as calculated on the basis of the molecular weights.

Sera

Freeze-dried, positive, syphilitic sera and fresh routine sera, positive and negative, the criterion for the latter being the Kahn standard test, were employed.

Methods

Quantitative complement-fixation test as modified by Mørch 1933

This method has recently been described by Schmidt [16] for use in experiments with cardiolipin antigen.

Serum dilution steps as used in the New York State Department of Health CF method [14]—i.e., parallel dilutions with a difference of 0.125 in logarithmic value between the tubes were employed. The results were given as $\log_{10}$ values at 50% haemolysis, determined by the Kärber method.[7] Preliminary complement (C′) titration experiments indicated that the same C′ titre could be used in experiments with the antigen containing synthetic lecithin as in experiments with the reference antigen.

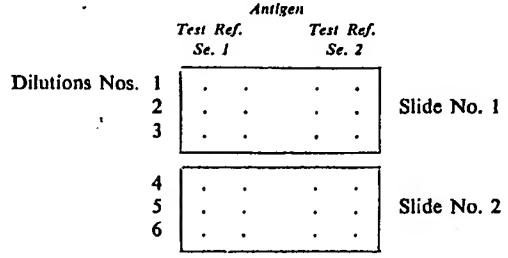
With the exception of the special maturation experiments described in this article, the saline antigen suspensions were always allowed to "mature" for two hours at room temperature.[12, 17] After that time the saline suspen-

sions of the test antigen, when examined with the naked eye, showed an opalescence similar to that of the reference antigen. All results were read by one technician and recorded by another.

VDRL slide flocculation test [20]

The readings were performed as in the CF experiments and the results were given as $\log_{10}$ titre values corresponding to " dils ".

In all experiments only one portion of each antigen was employed for all procedures; the experiments were all performed within four hours of the inactivation of sera. Antigens to be compared were dispensed as shown below:



In order to compare the sensitivity of the antigens as well as to test whether the relations between the reference and the test antigens were constant or not the following comparisons were made:

(1) CF test antigen versus CF reference antigen: experiments in February and April 1954.

(2) VDRL test antigen versus VDRL reference antigen: experiments in February, March, and April 1954.

In February 1954, furthermore, a maturation experiment [12, 17] was performed with the CF test antigen.

Results

CF experiments

120 fresh, routine, negative sera were found to be non-reactive with the test antigen as well as with the reference antigen.

(*a*) Five freeze-dried syphilitic sera were repeatedly tested with the reference antigen as well as with the test antigen on six experimental days, distributed over a period of two months.

In Table II a survey is given of the individual and average titres ($\log_{10}$ values) of the five sera as well as of the individual and average differences (d and $\bar{d}$) between the titres obtained with the two antigens. From this table it is evident that in general the titres were higher with the reference antigen than with the test antigen. The difference in titre varied considerably from one serum to another, serum 109 showing an average difference of

TABLE II. CF EXPERIMENTS

Date	Serum Nos. ...	109	114	118	119	120	Average difference ($\bar{d}$)
17.2.54	Test	2.593	2.450	1.734	1.830	1.957	
	Reference	2.593	2.625	1.734	1.941	2.084	
	Difference (d)	0.000	0.175	0.000	0.111	0.127	0.083
24.2.54	Test	2.672	2.545	1.830	2.148	2.179	
	Reference	2.688	2.768	1.925	2.243	2.259	
	Difference (d)	0.016	0.223	0.095	0.095	0.080	0.102
22.4.54	Test	2.354	2.418	1.591	1.718	1.845	
	Reference	2.466	2.545	1.671	1.877	1.989	
	Difference (d)	0.112	0.127	0.080	0.159	0.144	0.124
24.4.54	Test	2.529	2.370	1.639	1.782	1.893	
	Reference	2.625	2.529	1.718	1.957	1.989	
	Difference (d)	0.096	0.159	0.079	0.175	0.096	0.121
28.4.54	Test	2.354	2.338	1.527	1.766	1.845	
	Reference	2.434	2.545	1.639	1.893	1.893	
	Difference (d)	0.080	0.207	0.112	0.127	0.048	0.115
30.4.54	Test	2.497	2.450	1.702	1.845	1.989	
	Reference	2.561	2.577	1.766	2.020	2.068	
	Difference (d)	0.064	0.127	0.064	0.175	0.079	0.102
Average difference ($\bar{d}$)		0.061	0.170	0.072	0.140	0.096	0.108

Titration results for five freeze-dried syphilitic sera repeatedly tested in CF experiments with an antigen containing synthetic lecithin (test) and an antigen prepared with egg lecithin (reference). The titres are given as $\log_{10}$ values at 50 % haemolysis.

only 0.061 in comparison with serum 114, which showed an average difference of 0.170.

Statistical evaluation: An analysis of variance of the titre differences gave the following results:

Source of variation	*Sum of squares*		*Mean square*	v^2	
Sera	0.05099	4	$s_2^2 = 0.01275$	8.12	0.05%
Days	0.00603	5	$s_1^2 = 0.00121$	0.77	50%-70%
Residuals	0.03138	20	$s_0^2 = 0.00157$		
Total	0.08840	29			

The mean square corresponding to the day-by-day variations (s_1^2) is smaller than the mean square corresponding to the residual variation (s_0^2). Thus, no change in the difference between the reference and the test antigens was demonstrated during the observation period of two months.

As already mentioned, the average difference, $\bar{d}$, found for the five sera varied from 0.061 to 0.170. The result of a v^2-test comparing the mean square corresponding to the variation between sera with s_0^2, given in the test above, showed a marked significant difference.

The total average difference, being 0.108 with a standard error of

$$\frac{s_g}{\sqrt{30}} = 0.0206,$$

is clearly significant ($t = 5.02$, $P < 0.5\%$).

(*b*) *Maturation experiment.* The test antigen was tested against eight routine positive sera after 0, 20, 60, 120, and 24 × 60 minutes of maturation at room temperature; that is to say, the saline antigen suspensions were left standing for different periods before being added to the serum dilutions. The 24-hour-old suspension was prepared on the day before the experiment; all the other suspensions were derived from one and the same suspension prepared at t=0 minutes, maturation being interrupted by the addition of C′ to the portions of the suspension at different intervals.

From zero time to 20 minutes after the preparation of the antigen suspension all sera showed a rise in titre, averaging about 0.21 in $\log_{10}$ value. Thereafter, slightly increasing titres were found until two hours after the preparation, in total: 0.05 in $\log_{10}$ value. After 24 hours' maturation the average titre was about 0.08 less than after 2 hours' maturation, but it should be borne in mind that the 24 hours' suspension was prepared separately.

VDRL experiments

The negative reactions and the saline control for the test antigen were very similar to those obtained with the reference antigen, but the floccules in the positive reactions were not as big as those found with the reference antigen, floccules stronger than 2+ being rarely observed with the test antigen.

120 fresh, routine, negative sera were found to be non-reactive with the test antigen as well as with the reference antigen.

The reference antigen and the test antigen containing synthetic lecithin in an amount comparable to that of the natural lecithin in the reference antigen were repeatedly tested against six freeze-dried syphilitic sera on six experimental days, distributed over a period of two months. The individual and average titres, together with the corresponding differences (d and $\bar{d}$), are given in Table III.

As was the case in the CF experiments, the titres were higher with the reference antigen than with the test antigen, the average difference in this instance being greater.

Statistical evaluation. An analysis of variance of the titre differences gave the following results:

Source of variation	*Sum of squares*		*Mean square*	v^2	*P*
Sera	0.2416	5	$s_2^2 = 0.0483$	1.54	10%-30%
Days	0.2416	5	$s_1^2 = 0.0483$	1.54	10%-30%
Residuals	0.7852	25	$s_0^2 = 0.0314$		
Total	1.2684	35			

Neither the mean square corresponding to the variation between days (s_1^2) nor the mean square corresponding to the variation between sera (s_2^2) differs significantly from the residual variation (s_0^2), the latter being about 20 times as high as that found in the CF experiments.

The total average difference is 0.301, with a standard error of

$$\frac{s_g}{\sqrt{36}} = 0.037,$$

which gives a t value of 8.1 ($P < 0.05\%$). Thus, as in the CF experiments, a marked, significant total average difference is observed between the titres obtained with the reference antigen and those obtained with the test antigen.

Summary and Discussion

Crystalline, saturated L-α-dimyristoyl lecithin can replace natural lecithin in cardiolipin antigens. However, in both CF and VDRL experiments using the synthetic and natural lecithin in equimolar amounts the titres were significantly higher with the antigens containing natural lecithin than with the antigens prepared with synthetic lecithin. In the CF experiments the difference was comparatively small: about 0.10 in $\log_{10}$ value or corresponding to a ratio between titres of 1 : 1.25. In the VDRL

TABLE III. VDRL EXPERIMENTS

Date	Serum Nos....	118	119	120	121	122	123	Average difference $\bar{d}$
12.2.54	Test	0.903	0.903	0.602	0.301	0.602	0.301	
	Reference	0.903	1.204	0.602	0.602	0.602	0.602	
	Difference (d)	0.000	0.301	0.000	0.301	0.000	0.301	0.151
13.2.54	Test	0.602	0.602	0.301	0.000	0.301	0.301	
	Reference	1.204	1.204	0.602	0.301	0.602	0.301	
	Difference (d)	0.602	0.602	0.301	0.301	0.301	0.000	0.351
16.3.54	Test	0.602	0.602	0.301	0.301	0.301	0.000	
	Reference	0.903	1.204	0.602	0.301	0.602	0.301	
	Difference (d)	0.301	0.602	0.301	0.000	0.301	0.301	0.301
17.3.54	Test	0.903	0.903	0.301	0.301	0.301	0.301	
	Reference	1.204	1.204	0.602	0.602	0.903	0.602	
	Difference (d)	0.301	0.301	0.301	0.301	0.602	0.301	0.351
12.4.54	Test	0.602	0.301	0.000	0.000	0.301	0.000	
	Reference	0.903	0.903	0.602	0.301	0.602	0.301	
	Difference (d)	0.301	0.602	0.602	0.301	0.301	0.301	0.401
13.4.54	Test	0.301	0.602	0.301	0.301	0.301	0.000	
	Reference	0.903	0.903	0.602	0.301	0.301	0.301	
	Difference (d)	0.602	0.301	0.301	0.000	0.000	0.301	0.251
Average difference $\bar{d}$		0.351	0.452	0.301	0.201	0.251	0.251	0.301

Titration results for six freeze-dried syphilitic sera repeatedly tested in VDRL experiments with an antigen containing synthetic lecithin (test) and an antigen prepared with egg lecithin (reference). The titres are given as $\log_{10}$ values corresponding to "dils".

experiments the difference was greater; about 0.30 in $\log_{10}$ value or corresponding to a ratio between titres of 1 : 2.

In the VDRL experiments, the floccules of the test antigen differed from those of the reference antigen, generally being smaller with the test antigen than with the reference antigen; this is in conformity with Baer & Martin's observations.[4]

Within the two-month experimental period no change in the relative reactivity of the test and reference antigens was demonstrated. This is in contrast to the observation of Faure & Maréchal [6] with unpurified L-α-dimyristoyl lecithin that Kline antigen deteriorated in less than one week. However, in VDRL and CF experiments (the results of which are to be published shortly [a]) the present authors demonstrated that antigens containing synthetic lecithin showed a greater loss in sensitivity when exposed at 56° C for four months than did antigens containing purified, natural egg lecithin.

In the CF experiments it was demonstrated that different sera responded differently to the shift in antigens.

The maturation phenomenon [12, 17] was observed in saline suspensions of CF antigen prepared with synthetic lecithin.

The specificity of the test antigens was examined in 120 routine negative sera in both tests; no hypersensitivity was observed, all sera showing negative results in both tests.

Thus, with equimolar contents of synthetic and natural lecithin in the CF and VDRL antigens the "same serological reactions" were not obtained.

Kline [8, 9, 10] reports that in his test the cardiolipin/lecithin ratio (C/L ratio) should be altered from about 1C/9-10L, as used for the natural lecithins, to about 10C/9L when synthetic lecithin is used. Similarly Faure & Maréchal [6] observed that the sensitivity of the Kolmer and Kline antigens prepared with synthetic lecithin was increased with decreased C/L ratio. Hence, it is highly probable that the use of other concentrations of synthetic lecithin in the VDRL slide flocculation antigen, as well as in the CF antigen would result in increased sensitivity of these antigens.

The results of the experiments underline the necessity for further investigation before the natural purified lecithins are finally replaced by synthetic lecithin in the preparation of cardiolipin antigens.

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[a] Reyn, A., Bentzon, M. W. & Hartmann, J. (1956) Serological, nephelometrical and statistical studies on the employment of synthetic lecithin in cardiolipin antigen. *Brit. J. vener. Dis.* (in press)

RÉSUMÉ

La modification la plus récente apportée aux antigènes cardiolipidiques est l'emploi de lécithines synthétiques. Plusieurs types de ces substances ont été préparés, dont le degré d'activité sérologique est variable. Il a paru que l'un de ces produits, la lécithine L-α-dimyristique cristallisée pourrait remplacer dans la pratique les lécithines naturelles hautement purifiées.

Pour satisfaire aux exigences, la lécithine synthétique doit donner les mêmes résultats sérologiques que la lécithine naturelle et présenter la même stabilité à la conservation. Les auteurs ont procédé à des essais comparatifs en introduisant la lécithine L-α-dimyristique dans quatre antigènes: deux d'entre eux préparés en vue d'épreuves de fixation du complément, les deux autres en vue de tests de floculation sur lame VDRL.

Les résultats ont montré que la lécithine L-α-dimyristique synthétique pourrait remplacer la lécithine naturelle, mais que divers points doivent être éclaircis avant qu'elle soit introduite dans la pratique. A concentrations équimoléculaires, cette lécithine synthétique n'a pas donné des résultats sérologiques identiques à ceux des lécithines naturelles. D'autre part, après quatre mois de conservation à 56° C, les antigènes contenant la lécithine synthétique accusaient une baisse de sensibilité supérieure à celle des antigènes contenant des lécithines naturelles. La question du rapport Cardiolipine/Lécithine, dans les antigènes, reste à préciser. Kline, en effet, estime que pour l'exécution de son test, le rapport C/L, qui est de 1C/9-10L avec les lécithines naturelles, devrait être de 10C/9L avec la lécithine synthétique. Il est probable, d'après d'autres expériences encore, qu'en employant la lécithine synthétique à des concentrations différentes de celles que l'on utilise actuellement pour les lécithines naturelles, on accroîtrait la sensibilité des antigènes dans les tests de fixation du complément et de floculation sur lame.

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ÉTUDE DES PROPRIÉTÉS SÉROLOGIQUES DE LA LÉCITHINE L-α-DIMYRISTIQUE SYNTHÉTIQUE CRISTALLISÉE

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RÉSUMÉ

Les auteurs comparent un nouvel échantillon de lécithine L-α-dimyristique synthétique cristallisée avec la lécithine d'œuf qui sert habituellement à préparer les solutions antigéniques pour le sérodiagnostic de la syphilis.

Ils concluent que les solutions antigéniques à base de lécithine dimyristique cristallisée sont stables mais très légèrement moins sensibles que les solutions à base de lécithine d'œuf; on peut parfaitement bien les utiliser pour le sérodiagnostic de la syphilis.

Dans un travail précédent,[a] nous avons comparé les activités sérologiques de la lécithine d'œuf et d'un échantillon de lécithine L-α-dimyristique synthétique, que nous avait adressé le Professeur E. Baer en janvier 1951. Nous avions alors constaté que cette lécithine dimyristique donnait, lorsqu'on l'associait à de la cardiolipine et à du cholestérol, des antigènes moins sensibles que la lécithine d'œuf. Nous avions également remarqué que les solutions antigéniques renfermant cette lécithine synthétique se conservaient mal. Ce fait nous avait amenée à conclure en ces termes: « Il serait très souhaitable de disposer de produits synthétiques parfaitement définis pour préparer les antigènes destinés au sérodiagnostic de la syphilis, mais la lécithine dimyristique qui nous a été fournie nous ayant donné des solutions antigéniques instables par association avec le cardiolipide, il nous paraît prudent, dans l'état actuel de la question, de ne pas utiliser ce produit pour pratiquer ce sérodiagnostic. »

Etant donné le grand intérêt que présenterait l'utilisation de produits synthétiques définis, il nous a été demandé de reprendre cette étude sérologique avec un nouvel échantillon de lécithine L-α-dimyristique synthétique cristallisée que le Professeur Baer nous a fait parvenir en décembre 1953 à la demande de l'Organisation Mondiale de la Santé.

[a] Faure, M. (1952) *Ann. Inst. Pasteur*, 82, 738